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(57) Abstract

The invention relates to bone material for the prevention and treatment of osteomyelitis, which material is provided with antimicrobial peptides (AMPs) consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively charged amino acids and the majority of the amino acids of the other half of the domain are uncharged amino acids, which AMPs can be released to the surrounding area for a period of time and wherein the bone material forms bone cement after curing and the AMPs are distributed homogeneously in the cured bone cement. The invention further relates to a method of manufacturing the bone material, wherein the bone material is cured to bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement.

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BONE CEMENT WITH ANTIMICROBIAL PEPTIDES

The invention relates to the use of antimicrobial peptides (AMP) in calcium phosphate bone cement and forms a system which provides for slow release of the AMP for prevention and treatment of infections of the bone (osteomyelitis) and the surrounding soft tissues.

Preventing infections of the soft tissues and the bone after operations remains a cause for concern in orthopaedic and trauma surgery. Infection of bone tissues (osteomyelitis) and/or the surrounding soft tissue is very difficult to cure and this is a reason why stringent prevention is required. At this moment granules of polymethyl methacrylate (PMMA-granules) are used for this purpose. When they are placed in the surgical wound they function as a slow release system for obtaining high local concentrations of antibiotics, while the systemic concentrations remain low. Such granules are however non-re-absorbable and an additional operation is therefore necessary. The intensive use of antibiotics in human and veterinary medicine has further resulted in large scale resistance of bacteria and fungi to antibiotics such as gentamicin. New therapies for prevention and treatment of for instance osteomyelitis are therefore urgently required.

The present invention provides for this purpose a new system for the prevention and treatment of osteo-myelitis, which makes use of a re-absorbable calcium phosphate cement carrier and a new class of antibiotic agents, the so-called antimicrobial peptides (AMPs).

The AMPs used in the invention are peptides consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively

charged amino acids and the majority of the other half of the domain are uncharged amino acids.

The structure of these peptides has a number of variations. Firstly, the domain can form an α-helix, of which at least a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains a positively charged amino acid, position 8 is a positive or an uncharged amino acid and at least a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains an uncharged amino acid. These peptides have a lateral amphipathicity, i.e. a maximum hydrophobic moment at 100°. Stated simply, these peptides are hydrophobic on the left side and hydrophilic on the right side or vice versa. These peptides are referred to herein as "type I".

The domain can further form an α-helix, of which at least a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains an uncharged amino acid, position 8 is a positive or an uncharged amino acid and at least a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains a positively charged amino acid. These peptides have a lateral amphipathicity, i.e. a maximum hydrophobic moment at 100°. Stated simply, these peptides are hydrophobic on the right side and hydrophilic on the left side or vice versa. These peptides are designated "type II" herein and are in principle mirror-symmetrical to type I peptides.

In addition, the domain can form an α-helix, wherein at least a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains an uncharged amino acid and a positively charged amino acid is found at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25. These peptides have a longitudinal amphipathicity, i.e. a minimum hydrophobic moment at 100°. These peptides are hydrophobic on their "top" and hydrophilic on their "bottom". Such peptides are designated "type III".

Conversely, the domain can form an α-helix, wherein at least a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains a positively charged amino acid and an uncharged amino acid is found at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25. These peptides likewise have a longitudinal amphipathicity and therefore a minimum hydrophobic moment at 100°. These peptides are hydrophobic on their "bottom" and hydrophilic on their "top". Such peptides are designated "type IV".

Finally, the domain can form a so-called ß-strand and contain a positively charged amino acid on at least a majority of the positions 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21, 23 and 25) and an uncharged amino acid on at least a majority of the positions 2, 4, 6, 8, 10, (12, 14, 16, 18, 20, 22, 24). Such a ß-strand is laterally amphipathic and has a maximum hydrophobic moment at 180°. The ß-strand structure is flatter than the α-helix and, stated simply, is hydrophobic on the left and hydrophilic on the right or vice versa. These are "type V" peptides.

The positively charged amino acids are preferably chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H), while the uncharged amino acids are preferably chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W). Amino acids on the border between hydrophilic and hydrophobic can be chosen from both groups or from the remaining amino acids.

Hardly any difference in activity can in principle be detected when one of the positive amino acids and/or one of the uncharged amino acids is replaced by a random amino acid. The majority of the positively charged amino acids is therefore preferably the total number of

positively charged amino acids minus 1 and the majority of the uncharged amino acids is preferably the total number of uncharged amino acids minus 1.

The domain can be a part of a larger peptide but can itself also make up the entire peptide. When the domain forms part of a larger peptide, the C-terminal and/or N-terminal amino acids which are then additionally present can be random amino acids.

The following peptides of the type I are particularly recommended:

KRLFKELKFSLRKY (peptide 3)
KRLFKELLFSLRKY (peptide 4)
KRLFKELKKSLRKY (peptide 5)
KRLFKELLKSLRKY (peptide 6
OOLFOELOOSLOOY (peptide 7)
OOLFOELLOSLOOY (peptide 8)
KRLFKKLKFSLRKY (peptide 9)
KRLFKKLLFSLRKY (peptide 10)

A preferred peptide of the type III has the following amino acid sequence:

LLLFLLKKRKKRKY (peptide 11)

The peptides according to the invention can also contain further modifications. These modifications are for instance an N-terminal amide ring, for instance with acetic acid anhydride, or an alternative cleavage of the synthesis resin by which the C-terminus is modified. For this latter a replacement of the C-terminal carboxylic acid group by an amide, ester, ketone, aldehyde or alcohol group can be envisaged. Peptides with such a modification are for instance:

KRLFKELKFSLRKY-amide (peptide 12)
KRLFKELLFSLRKY-amide (peptide 13)

In addition to single peptides, oligomers can also be made. These are preferably linear oligomers of the peptides according to the invention. The coupling can be head-to-head and tail-to-tail as well as head-to-tail,

either by direct synthesis or by post-synthetic enzymatic coupling. For a trans-membrane pore formation a minimum peptide length is required. Oligomers of the peptides according to the invention are double length and thereby better able in principle to span the whole phospholipid double layer of the bacterial cell membrane at one time. The activity of the peptide could hereby improve even further. In addition, extension of the peptides provides stabilisation of the helix conformation. A spacer must usually be inserted. In direct synthesis of head-to-tail coupled oligomers a spacer can be inserted to size by the use of a chain of unnatural amino acids of the correct length, for instance ß-alanine, γ-amino butyric acid, ε-amino caproic acid, etc. Heterodifunctional coupling reagents, such as are commercially available for coupling peptide antigens to carrier proteins (for instance 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), m-maleimidobenzoyl)-N- hydroxysuccinimide ester (MBS), N-succinimidyl 3-[pyridyldithio]propionate (SPDD) etc.) are used to make linear oligomers with an inserted spacer. For head-to-head and tail-to-tail couplings can be used trivalent amino acids such as asparagine acid (D), glutamine acid (E), ornithine (O), lysine (K), serine (S), cysteine. Such oligomers are for instance:

KRKFHEKHHSHRGYC-CYGRHSHHKEHFKRK (peptide 14)

YGRHSHHKEHFKRKC-CKRKFHEKHHSHRGY (peptide 15)

$^{\alpha}N,^{\epsilon}N$-(KRKFHEKHHSHRGY)$_2$K-amide (peptide 16)

$^{\alpha}N,^{\epsilon}N$-(KRLFKELKFSLRKY)$_2$K-amide (peptide 17)

$^{\alpha}N,^{\epsilon}N$-(KRLFKKLKFSLRKY)$_2$K-amide (peptide 18)

Peptides 14 and 15 are obtained by synthesis of peptide 2 with an additional C-terminal respectively N-terminal cysteine, whereafter the oligomer is obtained by air oxidation. Peptides 16, 17 and 18 are obtained by making use of the Multiple Antigenic Peptide (MAP) strategy, wherein a lysine having on both the α- and on the ε-amino group an Fmoc protection was used as first amino acid on

the synthesis resin, whereby two identical amino acid chains (peptides 2, 3 and 9) were synthesized simultaneously on one lysine molecule.

The peptides described herein have no or hardly any haemolytic activity in physiological buffers such as PBS (phosphate-buffered saline solution). A low activity against erythrocytes of human origin is an indication of low toxicity. This selectivity is essential for the use of these peptides as antibiotics.

The peptides have a wide spectrum of antibacterial and antifungal activity, even against methycillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa (which is particularly dangerous in the case of osteomyelitis) and amphotericin-B-resistant Candida albicans.

The invention further makes use of bone material which after curing forms bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement. It is biocompatible, re-absorbable and inert, and forms at body temperature. The final cement moreover has sufficient strength and stiffness to serve as bone replacement.

It has been found according to the invention that the inclusion of the AMPs in the cement does not affect the mechanical properties thereof.

In order to include the AMPs in the cement, they are dissolved in a liquid medium, preferably water, and mixed with the bone material before or after curing thereof.

A blood protein-containing solution, in particular albumin, is preferably used to hold the AMPs in solution, in order to ensure a homogeneous distribution of the AMPs in the final cured bone cement.

In a preferred embodiment bone material contains calcium phosphate. With a view to the biocompatibility this is particularly a mixture of dicalcium phosphate,

tricalcium phosphate, tetracalcium phosphate and/or hydroxyl-apatite.

The invention further relates to a method of manufacturing a bone material according to the invention, wherein the bone material is cured to bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement. As stated, the AMPs are dissolved in a liquid medium, preferably water, and mixed with the bone material before or after curing thereof. The AMPs are preferably mixed with the bone material after curing. A longer release period is thus provided in which the AMPs can be released to the surrounding area after arranging of the bone material. The starting point here in each case is that the AMPs are always active only where this is necessary.

The invention also relates to a device for administering bone material provided with AMPs according to the invention, wherein provision is made for at least two compartments for separately containing the bone material and AMPs, a mixing chamber for mixing the bone material and the AMPs and a spray nozzle for spraying the mixture out of the mixing chamber.

The invention will be further elucidated with reference to a discussion of a number of tests in accordance with preferred variants of the invention, wherein the procedures for manufacturing the present bone material with added AMPs will be discussed.

1. A sterile cement powder consists of a mixture of alpha-tricalcium phosphate, tetracalcium phosphate-monoxide en dicalcium phosphate dibasic in a ratio of 75:20:5, or otherwise if desired.

2. A sterile AMP solution (solution (A)) consists of 4 mM HCl in water having dissolved therein 0.1% beef

or human serum albumin and AMPs in a concentration as required varying from $2x10^{-5}$% to 2%.

3. A second sterile solution (solution (B)) consists of water having dissolved therein 12% sodium succinate and 5% chondroitin succinate.

4. Solution (A) is mixed 1 to 1 with solution (B) under sterile conditions.

5. One volume part solution (A+B) is mixed with two volume parts cement powder under sterile conditions. This can take place:

a. in a dish and mixed with a spatula, whereafter the cement paste is arranged immediately in-situ in the body of the patient and there cures;

b. via a spray with two chambers, one of which contains the cement powder and the other solution (A+B); using the spray, powder and liquid are brought together in-situ in the body, whereafter the mixture cures at this location.

c. in a dish, mould or container, whereafter the mixture cures outside the body and is optionally ground to a powder of the desired granule size, whereafter it is arranged in the body of the patient.

6. One volume part solution B is mixed with two volume parts cement powder under sterile conditions in a dish, mould or container, whereafter the mixture cures and is ground to a

powder of the desired granule size. The cured cement is then incubated for 1 or more hours in solution A, whereafter the cement with absorbed AMPs is dried and stored in dry form until it is arranged in the body of the patient.

CLAIMS

1. Bone material for the prevention and treatment of osteomyelitis, which material is provided with antimicrobial peptides (AMPs) consisting of an amino acid chain which contains a domain of 10 to 25 amino acids, wherein the majority of the amino acids of the one half of the domain are positively charged amino acids and the majority of the amino acids of the other half of the domain are uncharged amino acids, which AMPs can be released to the surrounding area for a period of time and wherein the bone material forms bone cement after curing and the AMPs are distributed homogeneously in the cured bone cement.

2. Bone material as claimed in claim 1, **characterized in that** the domain forms an α-helix and at least at a majority of the positions 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 and 24) contains a positively charged amino acid, at position 8 a positive or an uncharged amino acid and at least at a majority of the positions 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) contains an uncharged amino acid.

3. Bone material as claimed in claim 2, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

4. Bone material as claimed in claim 2 or 3, **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

5. Bone material as claimed in claims 2-4, **characterized in that** the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

6. Bone material as claimed in claims 2-5, **characterized in that** the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

7. Bone material as claimed in claims 2-6, **characterized in that** the domain makes up the entire peptide.

8. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELKFSLRKY (peptide 3).

9. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELLFSLRKY (peptide 4).

10. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELKKSLRKY (peptide 5).

11. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKELLKSLRKY (peptide 6).

12. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

OOLFOELOOSLOOY peptide 7).

13. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

OOLFOELLOSLOOY (peptide 8).

14. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKKLKFSLRKY (peptide 9).

15. Bone material as claimed in claims 2-7, of which the domain has the following amino acid sequence:

KRLFKKLLFSLRKY (peptide 10).

16. Bone material as claimed in claim 1, **characterized in that** the domain forms an α-helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains an uncharged amino acid and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 a positively charged amino acid.

17. Bone material as claimed in claim 1, **characterized in that** the domain forms an α-helix and at least at a majority of the positions 1 to 6 (or 7 or 8 or 9 or 10 or 11 or 12) contains a positively charged amino acid and at position 7 (or 8 or 9 or 10 or 11 or 12 or 13) to 25 an uncharged amino acid.

18. Bone material as claimed in claim 16 or 17, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

19. Bone material as claimed in claim 16, 17 or 18, **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

20. Bone material as claimed in claims 16-19, **characterized in that** the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

21. Bone material as claimed in claims 16-20, **characterized in that** the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

22. Bone material as claimed in claims 16-21, **characterized in that** the domain makes up the entire peptide.

23. Bone material as claimed in claims 16 and 18-22, of which the domain has the following amino acid sequence:

LLLFLLKKRKKRKY (peptide 11).

24. Bone material as claimed in claim 1, **characterized in that** the domain forms a so-called ß-strand and contains a positively charged amino acid on at least a majority of the positions 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21, 23 and 25) and an uncharged amino acid on at least a majority of the positions 2, 4, 6, 8, 10, (12, 14, 16, 18, 20, 22, 24).

25. Bone material as claimed in claim 24, **characterized in that** the positively charged amino acids are chosen from the group consisting of ornithine (O), lysine (K), arginine (R) and histidine (H).

26. Bone material as claimed in claim 24, **characterized in that** the uncharged amino acids are chosen from the group consisting of the aliphatic amino acids glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), the amino acids with a dipolar side chain methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), the amino acids with an aromatic side chain phenylalanine (F), tyrosine (Y), tryptophan (W).

27. Bone material as claimed in claims 24-26, **characterized in that** the majority of the positively charged amino acids is the total number of positively charged amino acids minus 1.

28. Bone material as claimed in claims 24-27, **characterized in that** the majority of the uncharged amino acids is the total number of uncharged amino acids minus 1.

29. Bone material as claimed in claims 24-28, **characterized in that** the domain makes up the entire peptide.

30. Bone material as claimed in claims 1-29, wherein the N-terminus is amidated.

31. Bone material as claimed in claims 1-30, wherein the C-terminal carboxylic acid group is replaced by an amide, ester, ketone, aldehyde or alcohol group.

32. Method of manufacturing bone material as claimed in any of the claims 1-31, wherein the bone material is cured to bone cement and wherein the AMPs are distributed homogeneously in the cured bone cement.

33. Method as claimed in claim 32, wherein the AMPs are dissolved in liquid medium, preferably water, and mixed with the bone material after curing thereof.

34. Method as claimed in claim 32 or 33, wherein the cured bone cement is formed to a granulate.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00417

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L24/10 A61L27/22 A61K38/10 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39202 A (OSTEOGENICS INC) 12 December 1996 (1996-12-12) page 44, line 4; claims	1,32-34
A	EP 0 510 912 A (MORINAGA MILK INDUSTRY CO LTD) 28 October 1992 (1992-10-28) claims; examples	1-34
A	WO 97 18827 A (INTRABIOTICS PHARMACEUTICALS I) 29 May 1997 (1997-05-29) claims; examples	1
A	WO 94 15653 A (GENENTECH INC) 21 July 1994 (1994-07-21) claims	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1999	16/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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			DE	69223844 D	12-02-1998
			DE	69223844 T	16-04-1998
			DK	510912 T	09-02-1998
			JP	5148295 A	15-06-1993
			NZ	242437 A	27-07-1993
			US	5424396 A	13-06-1995
WO 9718827	A	29-05-1997	AU	704851 B	06-05-1999
			AU	1162997 A	11-06-1997
			AU	7739496 A	11-06-1997
			CA	2238429 A	29-05-1997
			CZ	9801591 A	14-10-1998
			CZ	9801592 A	16-12-1998
			EP	0862448 A	09-09-1998
			EP	0865292 A	23-09-1998
			HU	9901183 A	28-07-1999
			NO	982310 A	22-07-1998
			NO	982311 A	22-07-1998
			PL	326924 A	09-11-1998
			WO	9718826 A	29-05-1997
WO 9415653	A	21-07-1994	AT	153535 T	15-06-1997
			AU	671721 B	05-09-1996
			AU	6026294 A	15-08-1994
			CA	2151486 A	21-07-1994
			DE	69403439 D	03-07-1997
			DE	69403439 T	23-10-1997
			DK	679097 T	22-12-1997
			EP	0679097 A	02-11-1995
			ES	2105641 T	16-10-1997
			GR	3024277 T	31-10-1997
			JP	8505548 T	18-06-1996
			US	5422340 A	06-06-1995
WO 9420064	A	15-09-1994	US	5522893 A	04-06-1996
			AT	183382 T	15-09-1999
			AU	684722 B	08-01-1998
			AU	4923993 A	26-09-1994
			BR	9307825 A	14-11-1995
			CA	2157890 A,C	15-09-1994
			DE	69326082 D	23-09-1999
			EP	0688202 A	27-12-1995
			JP	8510713 T	12-11-1996
			US	5542973 A	06-08-1996
			US	5545254 A	13-08-1996
			US	5695729 A	09-12-1997
WO 9201462	A	06-02-1992	CA	2047317 A	20-01-1992
			JP	6504260 T	19-05-1994

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 99/00417

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201462 A		US 5294605 A	15-03-1994
WO 9937678 A	29-07-1999	NONE	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00417

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L24/10 A61L27/22 A61K38/10 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39202 A (OSTEOGENICS INC) 12 December 1996 (1996-12-12) page 44, line 4; claims	1,32-34
A	EP 0 510 912 A (MORINAGA MILK INDUSTRY CO LTD) 28 October 1992 (1992-10-28) claims; examples	1-34
A	WO 97 18827 A (INTRABIOTICS PHARMACEUTICALS I) 29 May 1997 (1997-05-29) claims; examples	1
A	WO 94 15653 A (GENENTECH INC) 21 July 1994 (1994-07-21) claims	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1999	16/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 20064 A (AMERICAN DENTAL ASS) 15 September 1994 (1994-09-15) claims; examples 1-10	1,32-34
A	DUCAN YU ET AL: "SELF-SETTING HYDROXYAPATITE CEMENT: A NOVEL SKELETAL DRUG-DELIVERY SYSTEM FOR ANTOBIOTICS" JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 81, no. 6, 1 June 1992 (1992-06-01), pages 529-531, XP000271282 ISSN: 0022-3549	1,32-34
A	WO 92 01462 A (SCRIPPS RESEARCH INST) 6 February 1992 (1992-02-06) claims	1
E	WO 99 37678 A (HELMERHORST EVA JOSEPHINE ;NIEUW AMERONGEN ARIE VAN (NL); STICHTIN) 29 July 1999 (1999-07-29) the whole document	1-34

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 99/00417

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9639202 A	12-12-1996	AU	6149696 A	24-12-1996
		BR	9608344 A	05-01-1999
		CA	2223596 A	12-12-1996
		EP	0830149 A	25-03-1998
		JP	11506659 T	15-06-1999
EP 0510912 A	28-10-1992	AU	664697 B	30-11-1995
		AU	1514692 A	29-10-1992
		CA	2066997 A	25-10-1992
		DE	69223844 D	12-02-1998
		DE	69223844 T	16-04-1998
		DK	510912 T	09-02-1998
		JP	5148295 A	15-06-1993
		NZ	242437 A	27-07-1993
		US	5424396 A	13-06-1995
WO 9718827 A	29-05-1997	AU	704851 B	06-05-1999
		AU	1162997 A	11-06-1997
		AU	7739496 A	11-06-1997
		CA	2238429 A	29-05-1997
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		EP	0865292 A	23-09-1998
		HU	9901183 A	28-07-1999
		NO	982310 A	22-07-1998
		NO	982311 A	22-07-1998
		PL	326924 A	09-11-1998
		WO	9718826 A	29-05-1997
WO 9415653 A	21-07-1994	AT	153535 T	15-06-1997
		AU	671721 B	05-09-1996
		AU	6026294 A	15-08-1994
		CA	2151486 A	21-07-1994
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		DK	679097 T	22-12-1997
		EP	0679097 A	02-11-1995
		ES	2105641 T	16-10-1997
		GR	3024277 T	31-10-1997
		JP	8505548 T	18-06-1996
		US	5422340 A	06-06-1995
WO 9420064 A	15-09-1994	US	5522893 A	04-06-1996
		AT	183382 T	15-09-1999
		AU	684722 B	08-01-1998
		AU	4923993 A	26-09-1994
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		CA	2157890 A,C	15-09-1994
		DE	69326082 D	23-09-1999
		EP	0688202 A	27-12-1995
		JP	8510713 T	12-11-1996
		US	5542973 A	06-08-1996
		US	5545254 A	13-08-1996
		US	5695729 A	09-12-1997
WO 9201462 A	06-02-1992	CA	2047317 A	20-01-1992
		JP	6504260 T	19-05-1994

IN NATIONAL SEARCH REPORT

Information on patent family members

National Application No
PCT/NL 99/00417

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201462 A		US 5294605 A	15-03-1994
WO 9937678 A	29-07-1999	NONE	

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
L/VQ54/cst/1		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL99/00417	02/07/1999	02/07/1998
International Patent Classification (IPC) or national classification and IPC A61L24/10		
Applicant STICHTING SKELETAL TISSUE ENGINEERING.... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/01/2000	16.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Cattell, James Telephone No. +49 89 2399 8468

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL99/00417

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-9 as originally filed

Claims, No.:

1-34 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-34
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-34
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-34
	No:	Claims	

2. Citations and explanations

see separate sheet

V.

1). Document D1 (WO96/39202) discloses on page 44 line 4 the use of peptide antibiotics in bone cement. The skilled man would be faced with the problem of selecting a suitable peptide.
Document D2 (WO92/01462) discloses such peptide (see peptide I) being active against P.aeroginosa and S.aureus (See table VII and compare with present application page 6 lines 10 to 15). D2 page 8 line 13 also mentions activity against C.albicans.

It has however been shown that the combination of the teachings of D1 and D2 has lead to unexpected release properties and beneficial antimicrobial action. The claims therefore meet the requirements of Article 33 PCT.

PCT

REQUEST

02.09.99

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/NL 99 / 00417

International Filing Date 02 JUL 1999 (02.07.99)

BUREAU VOOR DE INDUSTRIËLE EIGENDOM
P.C.T. INTERNATIONAL APPLICATION
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* L/VQ54/cst/1

Box No. I TITLE OF INVENTION

Bone cement with antimicrobial peptides

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Stichting Skeletal Tissue Engineering
Group Amsterdam
c/o Academisch Ziekenhuis Vrije Universiteit
De Boelelaan 1117
1081 HV AMSTERDAM

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: The Netherlands

State *(that is, country)* of residence: The Netherlands

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Burger, Elisabeth Henriëtte
Korteraarseweg 107
2461 GK TER AAR

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: The Netherlands

State *(that is, country)* of residence: The Netherlands

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Van Someren, Petronella Francisca Hendrika Maria
ARNOLD & SIEDSMA
Sweelinckplein 1
NL-2517 GK THE HAGUE
The Netherlands

Telephone No. 070 - 3654833

Facsimile No. 070 - 3452140

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

C ntinuation f Box No. I FURTHER APPLICANT(S) AND/OR (FURT) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Van Nieuw Amerongen, Arie
G. van Nijenrodestraat 136
3621 GK Breukelen
the Netherlands

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

RO

State *(that is, country)* of nationality: The Netherlands NL

State *(that is, country)* of residence: The Netherlands NL

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Wuisman, Paulus Ignatius Jozef Maria
Lupine Oord 29
3991 VG HOUTEN
the Netherlands

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: The Netherlands NL

State *(that is, country)* of residence: The Netherlands NL

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant f r the purposes f:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/ r (further) inventors are indicated n another continuati n sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ **AP** **ARIPO Patent:** **GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** **Eurasian Patent:** **AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ **AE** United Arab Emirates
- ☒ **AL** Albania
- ☒ **AM** Armenia
- ☒ **AT** Austria
- ☒ **AU** Australia
- ☒ **AZ** Azerbaijan
- ☒ **BA** Bosnia and Herzegovina
- ☒ **BB** Barbados
- ☒ **BG** Bulgaria
- ☒ **BR** Brazil
- ☒ **BY** Belarus
- ☒ **CA** Canada
- ☒ **CH and LI** Switzerland and Liechtenstein
- ☒ **CN** China
- ☒ **CU** Cuba
- ☒ **CZ** Czech Republic
- ☒ **DE** Germany
- ☒ **DK** Denmark
- ☒ **EE** Estonia
- ☒ **ES** Spain
- ☒ **FI** Finland
- ☒ **GB** United Kingdom
- ☒ **GD** Grenada
- ☒ **GE** Georgia
- ☒ **GH** Ghana
- ☒ **GM** Gambia
- ☒ **HR** Croatia
- ☒ **HU** Hungary
- ☒ **ID** Indonesia
- ☒ **IL** Israel
- ☒ **IN** India
- ☒ **IS** Iceland
- ☒ **JP** Japan
- ☒ **KE** Kenya
- ☒ **KG** Kyrgyzstan
- ☒ **KP** Democratic People's Republic of Korea
- ☒ **KR** Republic of Korea
- ☒ **KZ** Kazakhstan
- ☒ **LC** Saint Lucia
- ☒ **LK** Sri Lanka
- ☒ **LR** Liberia
- ☒ **LS** Lesotho
- ☒ **LT** Lithuania
- ☒ **LU** Luxembourg
- ☒ **LV** Latvia
- ☒ **MD** Republic of Moldova
- ☒ **MG** Madagascar
- ☒ **MK** The former Yugoslav Republic of Macedonia
- ☒ **MN** Mongolia
- ☒ **MW** Malawi
- ☒ **MX** Mexico
- ☒ **NO** Norway
- ☒ **NZ** New Zealand
- ☒ **PL** Poland
- ☒ **PT** Portugal
- ☒ **RO** Romania
- ☒ **RU** Russian Federation
- ☒ **SD** Sudan
- ☒ **SE** Sweden
- ☒ **SG** Singapore
- ☒ **SI** Slovenia
- ☒ **SK** Slovakia
- ☒ **SL** Sierra Leone
- ☒ **TJ** Tajikistan
- ☒ **TM** Turkmenistan
- ☒ **TR** Turkey
- ☒ **TT** Trinidad and Tobago
- ☒ **UA** Ukraine
- ☒ **UG** Uganda
- ☒ **US** United States of America
- ☒ **UZ** Uzbekistan
- ☒ **VN** Viet Nam
- ☒ **YU** Yugoslavia
- ☒ **ZA** South Africa
- ☒ **ZW** Zimbabwe

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:

- ☐ .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claim indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 2 July 1998 (02.07.98)	98.202233.7 (EP)		Europe (EP)	
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*: ISA / EP

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:
Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	: 4
description (excluding sequence listing part)	: 9
claims	: 4
abstract	: 1
drawings	:
sequence listing part of description	:
Total number of sheets	: 18

This international application is **accompanied** by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: Dutch

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Bruin, Cornelis Willem
for
Van Someren, Petronella Francisca Hendrika Maria

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BOTCEMENT MET ANTIMICROBIËLE PEPTIDEN

De uitvinding heeft betrekking op het gebruik van antimicrobiële peptiden (AMP) in calciumfosfaat botcement en vormt een inrichting, die de vertraagde afgifte van de AMP verzorgt ter voorkoming en behandeling van infecties van het bot (osteomyelitis) en de omringende zachte weefsels.

Het voorkomen van infecties van de zachte weefsels en het bot na operaties blijft een zorgkwestie bij orthopedische en traumachirurgie. Infectie van botweefsels (osteomyelitis) en/of het omliggende zachte weefsel is zeer moeilijk te genezen en dit is een reden waarom een stringente preventie vereist is. Op dit moment worden hiervoor met antibiotica geïmpregneerde polymethylmethacrylaatkorrels (PMMA-korrels) toegepast. Wanneer zij in de chirurgische wond geplaatst zijn fungeren zij als een vertraagde afgifte systeem teneinde hoge lokale concentraties aan antibiotica te verkrijgen, terwijl de systemische concentraties laag blijven. Dergelijke korrels zijn echter niet-resorbeerbaar en daarom is een aanvullende operatie nodig. Verder heeft het intensieve gebruik van antibiotica in de humane en veterinaire geneeskunde geleid tot een resistentie op grote schaal van bacteriën en schimmels tegen antibiotica als gentamycine. Daarom zijn dringend nieuwe therapieën voor de voorkoming en behandeling van bijvoorbeeld osteomyelitis nodig.

De onderhavige uitvinding verschaft daartoe een nieuwe inrichting voor de voorkoming en behandeling van osteomyelitis, welke gebruik maakt van een resorbeerbare calciumfosfaatcement drager en een nieuwe klasse van antibiotische middelen, de zogeheten antimicrobiële peptiden (AMPs).

De AMPs, die worden gebruikt in de uitvinding zijn peptiden, die bestaan uit een aminozuurketen, welke een domein van 10 tot 25 aminozuren bevatten, waarbij de meerderheid van de aminozuren van de ene helft van het domein positief geladen aminozuren zijn en de meerderheid

van de andere helft van het domein ongeladen aminozuren zijn.

De opbouw van deze peptiden kent een aantal variaties. Ten eerste kan het domein een α-helix vormen, waarvan tenminste een meerderheid van de posities 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 en 24) een positief geladen aminozuur bevat, positie 8 een positief of een ongeladen aminozuur is en tenminste een meerderheid van de posities 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) een ongeladen aminozuur bevat. Deze peptiden hebben een laterale amfipathiciteit, dat wil zeggen een maximaal hydrofoob moment bij 100°. Eenvoudig gezegd, zijn deze peptiden aan de linkerzijde hydrofoob en aan de rechterzijde hydrofiel of omgekeerd. Deze peptiden worden hierin "type I" genoemd.

Verder kan het domein een α-helix vormen, waarvan tenminste een meerderheid van de posities 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 en 24) een ongeladen aminozuur bevat, positie 8 een positief of een ongeladen aminozuur is en tenminste een meerderheid van de posities 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) een positief geladen aminozuur bevat. Deze peptiden hebben een laterale amfipathiciteit, dat wil zeggen een maximaal hydrofoob moment bij 100°. Eenvoudig gezegd, zijn deze peptiden aan de rechterzijde hydrofoob en aan de linkerzijde hydrofiel of omgekeerd. Deze peptiden worden hierin "type II" genoemd en zijn in principe de spiegelbeelden van type I peptiden.

Daarnaast kan het domein een α-helix vormen, waarbij tenminste een meerderheid van de posities 1 tot en met 6 (of 7 of 8 of 9 of 10 of 11 of 12) een ongeladen aminozuur bevat, en op positie 7 (of 8 of 9 of 10 of 11 of 12 of 13) tot en met 25 een positief geladen aminozuur voorkomt. Deze peptiden hebben een langsgerichte amfipathiciteit, dat wil zeggen een minimaal hydrofoob moment bij 100°. Deze peptiden zijn aan hun "bovenkant" hydrofoob en aan hun "onderkant" hydrofiel. Dergelijke peptiden worden met "type III" aangeduid.

Omgekeerd kan het domein een α-helix vormen, waarbij tenminste een meerderheid van de posities 1 tot en met 6 (of 7 of 8 of 9 of 10 of 11 of 12) een positief geladen aminozuur bevat, en op positie 7 (of 8 of 9 of 10 of 11 of 12 of 13) tot en met 25 een ongeladen aminozuur voorkomt. Deze peptiden hebben eveneens een langsgerichte amfipathiciteit, en dus een minimaal hydrofoob moment bij 100°. Deze peptiden zijn aan hun "onderkant" hydrofoob en aan hun "bovenkant" hydrofiel. Dergelijke peptiden worden met "type IV" aangeduid.

Tenslotte kan het domein een zogeheten ß-strand vormen en op tenminste een meerderheid van de posities 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21, 23 en 25) een positief geladen aminozuur bevatten, en op tenminste een meerderheid van de posities 2, 4, 6, 8, 10, (12, 14, 16, 18, 20, 22, 24) een ongeladen aminozuur. Een dergelijke ß-strand is lateraal amfipathisch en heeft een maximaal hydrofoob moment bij 180°. De ß-strand structuur is vlakker dan de α-helix en is eenvoudig gezegd links hydrofoob en rechts hydrofiel of omgekeerd. Dit zijn "type V"-peptiden.

De positief geladen aminozuren worden bij voorkeur gekozen uit de groep, die bestaat uit ornithine (O), lysine (K), arginine (R) en histidine (H), terwijl de ongeladen aminozuren bij voorkeur worden gekozen uit de groep, die bestaat uit de alifatische aminozuren glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), de aminozuren met een dipolaire zijketen methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), de aminozuren met een aromatische zijketen phenylalanine (F), tyrosine (Y), tryptofaan (W). Aminozuren op de grens tussen hydrofiel en hydrofoob kunnen uit beide groepen of uit de overige aminozuren gekozen worden.

In principe is er nauwelijks verschil in activiteit waar te nemen wanneer een van de positieve aminozuren en/of een van de ongeladen aminozuren is vervangen door een willekeurig aminozuur. De meerderheid van de

positief geladen aminozuren is derhalve bij voorkeur het totale aantal positief geladen aminozuren minus 1 en de meerderh id van de ongeladen aminozuren is bij voorkeur het totale aantal ongeladen aminozuren minus 1.

Het domein kan een onderdeel zijn van een groter peptide, maar kan ook zelf het gehele peptide uitmaken. Wanneer het domein onderdeel uitmaakt van een groter peptide kunnen de C-terminale en/of N-terminale aminozuren, die dan extra aanwezig zijn willekeurige aminozuren zijn.

De bijzondere voorkeur gaat uit naar de volgende peptiden van het type I:

KRLFKELKFSLRKY	(peptide 3)
KRLFKELLFSLRKY	(peptide 4)
KRLFKELKKSLRKY	(peptide 5)
KRLFKELLKSLRKY	(peptide 6
OOLFOELOOSLOOY	(peptide 7)
OOLFOELLOSLOOY	(peptide 8)
KRLFKKLKFSLRKY	(peptide 9)
KRLFKKLLFSLRKY	(peptide 10)

Een voorkeurspeptide van het type III heeft de volgende aminozuurvolgorde:

LLLFLLKKRKKRKY	(peptide 11)

De peptiden volgens de uitvinding kunnen verder nog modificaties bevatten. Deze modificaties zijn bijvoorbeeld een N-terminale amidering, bijvoorbeeld met azijnzuuranhydride, of een alternatieve afsplitsing van de synthesehars, waardoor de C-terminus gemodificeerd wordt. Voor dit laatste kan gedacht worden aan een vervanging van de C-terminale carbonzuurgroep door een amide-, ester-, keton-, aldehyde- of alcoholgroep. Peptiden met een dergelijke modificatie zijn bijvoorbeeld:

KRLFKELKFSLRKY-amide	(peptide 12)
KRLFKELLFSLRKY-amide	(peptide 13)

Naast enkelvoudige peptiden kunnen ook oligomeren worden gemaakt. Dit zijn bij voorkeur lineaire oligomeren van de peptiden volgens de uitvinding. De koppeling kan zowel kop-kop als staart-staart als kop-staart zijn,

hetzij door directe synthese hetzij door postsynthetische enzymatische koppeling. Voor de vorming van een transmembraan porie is een minimale peptidenlengte vereist. Oligomeren van de peptiden volgens de uitvinding zijn dubbel lang en daardoor in principe beter in staat de gehele fosfolipide dubbellaag van de bacteriële celmembraan in een keer te overspannen. Hierdoor zou de werking van het peptide nog verder kunnen verbeteren. Daarnaast geeft verlenging van de peptiden een stabilisatie van de helixconformatie. Meestal dient een spacer te worden ingevoegd. Bij directe synthese van kop-staart gekoppelde oligomeren kan een spacer op maat worden ingebouwd door het gebruik van een keten van onnatuurlijke aminozuren van de juiste lengte, bijvoorbeeld ß-alanine, γ-aminoboterzuur, ε-aminocapronzuur, etc. Ook kunnen heterodifunctionele koppelingsreagentia, zoals die commercieel verkrijgbaar zijn om peptide-antigenen aan dragereiwitten te koppelen (bijvoorbeeld 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), m-maleimidobenzoyl)-N- hydroxysuccinimide ester (MBS), N-succinimidyl 3-[pyridyldithio]propionaat (SPDD) etc. gebruikt worden om lineaire oligomeren met een tussengevoegde spacer te maken. Voor kop-kop en staart-staartkoppelingen kunnen driewaardige aminozuren worden gebruikt, zoals asparaginezuur (D), glutaminezuur (E), ornithine (O), lysine (K), serine (S), cysteïne. Dergelijke oligomeren zijn bijvoorbeeld:

KRKFHEKHHSHRGYC-CYGRHSHHKEHFKRK (peptide 14)

YGRHSHHKEHFKRKC-CKRKFHEKHHSHRGY (peptide 15)

$^{\alpha}N,^{\epsilon}N$-$(KRKFHEKHHSHRGY)_2K$-amide (peptide 16)

$^{\alpha}N,^{\epsilon}N$-$(KRLFKELKFSLRKY)_2K$-amide (peptide 17)

$^{\alpha}N,^{\epsilon}N$-$(KRLFKKLKFSLRKY)_2K$-amide (peptide 18)

Peptiden 14 en 15 zijn verkregen door synthese van peptide 2 met een additionele C-terminale, respectievelijk N-terminale cysteïne, waarna het oligomeer verkregen is door luchtoxidatie. Peptiden 16, 17 en 18 zijn verkregen door gebruikmaking van de Multi-Antigen Peptide (MAP) strategie, waarbij als eerste aminozuur een lysine met zowel op de α- als op de ε-aminogroep een Fmoc bescher-

ming als eerste aminozuur aan d synthesehars werd gebruikt, waardoor gelijktijdig twee identieke aminozuurketens (peptiden 2, 3 n 9) op één lysinemolecuul werden gesynthetiseerd.

De hierin beschreven peptiden hebben geen of nauwelijks hemolytische activiteit in fysiologische buffers, zoals PBS (fosfaatgebufferde zoutoplossing). Een lage activiteit tegen erytrocyten van humane oorsprong is een indicatie voor lage toxiciteit. Deze selectiviteit is essentieel voor het gebruik van deze peptiden als antibiotica.

De peptiden vertonen een wijd spectrum aan antibacteriële en anti-schimmel activiteit, zelfs tegen methycilline-resistente Staphylococcus aureus (MRSA), Pseudomonas aeruginosa (welke in het bijzonder gevaarlijk is bij osteomyelitis) en amphotericine-B resistente Candida albicans.

De uitvinding maakt verder gebruik van botmateriaal dat na uitharding botcement vormt en waarbij de AMPs homogeen in het uitgeharde botcement zijn verdeeld. Het is biocompatibel, resorbeerbaar en inert en vormt zich bij lichaamstemperatuur. Het uiteindelijke cement heeft bovendien voldoende sterkte en stijfheid om als botvervanger dienst te doen.

Volgens de uitvinding is gevonden dat de opname van de AMPs in het cement niet van invloed zijn op de mechanische eigenschappen daarvan.

Teneinde de AMPs in het cement op te nemen worden zij opgelost in een vloeibaar medium, bij voorkeur water, met het botmateriaal vóór of na harding daarvan gemengd.

Bij voorkeur wordt een bloedeiwit bevattende oplossing, in het bijzonder albumine, gebruikt om de AMPs in oplossing te houden teneinde een homogene verdeling van de AMPs in het uiteindelijk uitgeharde botcement te waarborgen.

In een voorkeursuitvoeringsvorm van botmateriaal bevat het calciumfosfaat. Vanwege de biocompatibili-

teit is daarbij in het bijzonder sprake van een mengsel van di-calciumfosfaat, tri-calciumfosfaat, tetra-calciumfosfaat en/of hydroxyl-apatiet.

De uitvinding heeft verder betrekking op een werkwijze ter vervaardiging van een botmateriaal volgens de uitvinding, waarbij het botmateriaal wordt uitgehard tot botcement en waarbij de AMPs homogeen in het uitgeharde botcement wordt verdeeld. Zoals gezegd, worden de AMPs in een vloeibaar medium, bij voorkeur water, opgelost en met het botmateriaal vóór of na uitharding daarvan gemengd. Bij voorkeur worden de AMPs na uitharding met het botmateriaal gemengd. Aldus wordt bewerkstelligd dat er een langere afgifteperiode wordt verschaft, waarin de AMPs na aanbrenging van het botmateriaal kunnen worden afgegeven aan de omgeving. Daarbij is telkens het uitgangspunt dat de AMPs steeds alleen daar werken waar dat nodig is.

De uitvinding is tevens gerelateerd aan een inrichting ter toediening van botmateriaal voorzien van AMPs overeenkomstig de uitvinding, waarbij is voorzien in ten minste twee compartimenten voor het afzonderlijk houden van het botmateriaal en AMPs, in een mengkamer voor het mengen van het botmateriaal en de AMPs, alsmede in een spuitmond voor het vanuit de mengkamer spuiten van het mengsel.

De uitvinding zal nader worden toegelicht aan de hand van een bespreking van een aantal proeven volgens voorkeursvarianten van de uitvinding, waarbij de procedures voor het vervaardigen van het onderhavige botmateriaal met toegevoegde AMPs zullen worden besproken.

1. Een steriel cementpoeder bestaat uit een mengsel van alfa-tricalciumfosfaat, tetracalciumfosfaat-monoxyde en di-calciumfosfaat-dibasisch, in een verhouding van 75:20:5, of anders indien gewenst.

2. Een steriel AMP oplossing (oplossing (A)) bestaat uit 4 millimolair HCl in water met daarin opgelost 0,1% runder- of humaan serumalbumine en AMPs in een concentratie naar wens variërend van $2x10^{-5}$ % tot 2%.

3. Een tweede steriele oplossing (oplossing (B)) bestaat uit water met daarin opgelost 12% natriumsuccinaat en 5% chondroïtine-succinaat.

4. Oplossing (A) wordt 1 op 1 gemengd met oplossing (B), onder steriele omstandigheden.

5. Een volumedeel oplossing (A+B) wordt gemengd met twee volumedelen cementpoeder onder steriele omstandigheden. Dit kan gebeuren;

   a. in een bakje en gemengd met een spatel, waarna de cementpasta onmiddellijk in het lichaam van de patiënt ter plaatse wordt aangebracht en daar uithardt;

   b. via een spuit met twee kamers, waarvan er één het cementpoeder en de andere oplossing (A+B) bevat; met behulp van de spuit worden poeder en vloeistof ter plekke in het lichaam samengebracht waarna het mengsel ter plaatse uithardt.

   c. in een bak, mal of container, waarna het mengsel buiten het lichaam uithardt en al of niet wordt vermalen tot een poeder van de gewenste korrelgrootte, waarna het in het lichaam van de patiënt wordt aangebracht.

6. Een volumedeel oplossing B wordt gemengd met twee volumedelen cementpoeder onder steriele omstandigheden, in een bak, mal of container, waarna h t mengsel uithardt en wordt vermalen tot een poeder van de gewenste poedergrootte.

Het uitgeharde cement wordt vervolgens gedurende 1 of meerdere uren geïncubeerd in oplossing A, waarna het c ment met geabsorbeerde AMPs wordt gedroogd en in droge vorm wordt bewaard totdat het in het lichaam van de patient wordt aangebracht.

CONCLUSIES

1. Botmateriaal ter voorkoming en behandeling van osteomyelitis, welk materiaal is voorzien van antimicrobiële peptiden (AMPs), bestaande uit een aminozuurketen, welke een domein van 10 tot 25 aminozuren bevatten, waarbij de meerderheid van de aminozuren van de ene helft van het domein positief geladen aminozuren zijn en de meerderheid van de aminozuren van de andere helft van het domein ongeladen aminozuren zijn, welke AMPs gedurende een tijdsperiode aan de omgeving kunnen worden afgegeven, en waarbij het botmateriaal na uitharding botcement vormt en de AMPs homogeen in het uitgeharde botcement zijn verdeeld.

2. Botmateriaal volgens conclusie 1 **met het kenmerk** dat het domein een α-helix vormt en op tenminste een meerderheid van de posities 1, 2, 5, 6, 9 (12, 13, 16, 19, 20, 23 en 24) een positief geladen aminozuur, op positie 8 een positief of een ongeladen aminozuur en op tenminste een meerderheid van de posities 3, 4, 7, 10, (11, 14, 15, 17, 18, 21, 22, 25) een ongeladen aminozuur bevat.

3. Botmateriaal volgens conclusie 2 **met het kenmerk** dat de positief geladen aminozuren zijn gekozen uit de groep, die bestaat uit ornithine (O), lysine (K), arginine (R) en histidine (H).

4. Botmateriaal volgens conclusie 2 of 3, **met het kenmerk**, dat de ongeladen aminozuren zijn gekozen uit de groep, die bestaat uit de alifatische aminozuren glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), de aminozuren met een dipolaire zijketen methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), de aminozuren met een aromatische zijketen phenylalanine (F), tyrosine (Y), tryptofaan (W).

5. Botmateriaal volgens conclusies 2-4, **met het k nm rk**, dat de meerderheid van de positief geladen aminozuren het totale aantal positief geladen aminozuren minus 1 is.

6. Botmateriaal volgens conclusies 2-5, **met het kenmerk**, dat de meerderheid van de ongeladen aminozuren het totale aantal ongeladen aminozuren minus 1 is.

7. Botmateriaal volgens conclusies 2-6, **m t het kenmerk**, dat het domein het gehele peptide uitmaakt.

8. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

KRLFKELKFSLRKY (peptide 3).

9. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

KRLFKELLFSLRKY (peptide 4).

10. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

KRLFKELKKSLRKY (peptide 5).

11. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

KRLFKELLKSLRKY (peptide 6).

12. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

OOLFOELOOSLOOY (peptide 7).

13. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

OOLFOELLOSLOOY (peptide 8).

14. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

KRLFKKLKFSLRKY (peptide 9).

15. Botmateriaal volgens conclusies 2-7, waarvan het domein de volgende aminozuurvolgorde heeft:

KRLFKKLLFSLRKY (peptide 10).

16. Botmateriaal volgens conclusie 1, **met het kenmerk**, dat het domein een α-helix vormt en op tenminste een meerderheid van de posities 1 tot en met 6 (of 7 of 8 of 9 of 10 of 11 of 12) een ongeladen aminozuur bevat, en op positie 7 (of 8 of 9 of 10 of 11 of 12 of 13) tot en met 25 een positief geladen aminozuur.

17. Botmateriaal volgens conclusie 1, **m t h t kenm rk**, dat het domein een α-helix vormt en tenminste op

een meerderheid van de posities 1 tot en met 6 (of 7 of 8 of 9 of 10 of 11 of 12) een positief geladen aminozuur bevat, en op positie 7 (of 8 of 9 of 10 of 11 of 12 of 13) tot en met 25 een ongeladen aminozuur.

18. Botmateriaal volgens conclusie 16 of 17, **met het kenmerk**, dat de positief geladen aminozuren zijn gekozen uit de groep, die bestaat uit ornithine (O), lysine (K), arginine (R) en histidine (H).

19. Botmateriaal volgens conclusie 16, 17 of 18, **met het kenmerk**, dat de ongeladen aminozuren zijn gekozen uit de groep, die bestaat uit de alifatische aminozuren glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), de aminozuren met een dipolaire zijketen methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), de aminozuren met een aromatische zijketen phenylalanine (F), tyrosine (Y), tryptofaan (W).

20. Botmateriaal volgens conclusies 16-19, **met het kenmerk**, dat de meerderheid van de positief geladen aminozuren het totale aantal positief geladen aminozuren minus 1 is.

21. Botmateriaal volgens conclusies 16-20, **met het kenmerk**, dat de meerderheid van de ongeladen aminozuren het totale aantal ongeladen aminozuren minus 1 is.

22. Botmateriaal volgens conclusies 16-21, **met het kenmerk**, dat het domein het gehele peptide uitmaakt.

23. Botmateriaal volgens conclusies 16 en 18-22, waarvan het domein de volgende aminozuurvolgorde heeft:

LLLFLLKKRKKRKY (peptide 11).

24. Botmateriaal volgens conclusie 1, **met het kenmerk**, dat het domein een zogeheten ß-strand vormt en op tenminste een meerderheid van de posities 1, 3, 5, 7, 9 (11, 13, 15, 17, 19, 21, 23 en 25) een positief geladen aminozuur bevat, en op tenminste een meerderheid van de posities 2, 4, 6, 8, 10, (12, 14, 16, 18, 20, 22, 24) een ongeladen aminozuur.

25. Botmateriaal volgens conclusie 24, **met h t kenm rk**, dat de positief geladen aminozuren zijn gekozen uit de groep, die bestaat uit ornithine (O), lysine (K), arginine (R) en histidine (H).

26. Botmateriaal volgens conclusie 24, **met het kenmerk**, dat de ongeladen aminozuren zijn gekozen uit de groep, die bestaat uit de alifatische aminozuren glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), de aminozuren met een dipolaire zijketen methionine (M), asparagine (N), glutamine (Q), serine (S), threonine (T), de aminozuren met een aromatische zijketen phenylalanine (F), tyrosine (Y), tryptofaan (W).

27. Botmateriaal volgens conclusies 24-26, **met het kenmerk**, dat de meerderheid van de positief geladen aminozuren het totale aantal positief geladen aminozuren minus 1 is.

28. Botmateriaal volgens conclusies 24-27, **met het kenmerk**, dat de meerderheid van de ongeladen aminozuren het totale aantal ongeladen aminozuren minus 1 is.

29. Botmateriaal volgens conclusies 24-28, **met het kenmerk**, dat het domein het gehele peptide uitmaakt.

30. Botmateriaal volgens conclusies 1-29, waarbij de N-terminus geamideerd is.

31. Botmateriaal volgens conclusies 1-30, waarbij de C-terminale carbonzuurgroep is vervangen door een amide-, ester-, keton-, aldehyde- of alcoholgroep.

32. Werkwijze ter vervaardiging van botmateriaal volgens één van de conclusies 1-32, waarbij het botmateriaal wordt uitgehard tot botcement en waarbij de AMPs homogeen in het uitgeharde botcement worden verdeeld.

33. Werkwijze volgens conclusie 32, waarbij de AMPs in vloeibaar medium, bij voorkeur water, worden opgelost en met het botmateriaal na harding daarvan worden gemengd.

34. Werkwijze volgens conclusie 32 of 33, waarbij het uitgeharde botcement tot een granulaat wordt gevormd.

UITTREKSEL

De uitvinding heeft betrekking op botmateriaal ter voorkoming en behandeling van osteomyelitis, welk materiaal is voorzien van antimicrobiële peptiden (AMPs), bestaande uit een aminozuurketen, welke een domein van 10 tot 25 aminozuren bevatten, waarbij de meerderheid van de aminozuren van de ene helft van het domein positief geladen aminozuren zijn en de meerderheid van de aminozuren van de andere helft van het domein ongeladen aminozuren zijn, welke AMPs gedurende een tijdsperiode aan de omgeving kunnen worden afgegeven, en waarbij het botmateriaal na uitharding botcement vormt en de AMPs homogeen in het uitgeharde botcement zijn verdeeld. De uitvinding heeft verder betrekking op een werkwijze ter vervaardiging van het botmateriaal, waarbij het botmateriaal wordt uitgehard tot botcement en waarbij de AMPs homogeen in het uitgeharde botcement worden verdeeld.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
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PCT/NL99/00417	02/07/1999	02/07/1998
International Patent Classification (IPC) or national classification and IPC A61L24/10		
Applicant STICHTING SKELETAL TISSUE ENGINEERING.... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/01/2000	16.10.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Cattell, James Telephone No. +49 89 2399 8468

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL99/00417

I. Basis of th rep rt

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-9 as originally filed

Claims, No.:

1-34 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-34
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-34
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-34
	No:	Claims

2. Citations and explanations

see separate sheet

V.

1). Document D1 (WO96/39202) discloses on page 44 line 4 the use of peptide antibiotics in bone cement. The skilled man would be faced with the problem of selecting a suitable peptide.
Document D2 (WO92/01462) discloses such peptide (see peptide I) being active against P.aeroginosa and S.aureus (See table VII and compare with present application page 6 lines 10 to 15). D2 page 8 line 13 also mentions activity against C.albicans.

It has however been shown that the combination of the teachings of D1 and D2 has lead to unexpected release properties and beneficial antimicrobial action. The claims therefore meet the requirements of Article 33 PCT.

Form PTO-1390 (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	702-002201
	U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/720933

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATES CLAIMED
PCT/NL99/00417	02.07.99 (July 2, 1999)	02.07.98 (July 2, 1998)

TITLE OF INVENTION **BONE CEMENT WITH ANTIMICROBIAL PEPTIDES**

APPLICANT(S) FOR DO/EO/US **Elisabeth H. BURGER, Arie VAN NIEUW AMERONGEN, Paulus I. J. M. WUISMAN**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:
    a. WO 00/01427-Front Page with Abstract, specification and claims (15 pp.)
    b. Search Report (4 pp.)
    c. International Preliminary Examination Report (4 pp.)

526 RC'D PCT/PTO 02 JAN 2001

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/720933	INTERNATIONAL APPLICATION NO. PCT/NL99/00417	ATTORNEY'S DOCKET NUMBER 702-002201

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

		CALCULATIONS PTO USE ONLY
Search Report has been prepared by the EPO or JPO	$860.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$690.00	
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$710.00	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$1000.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$100.00	

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	34 - 20	14	X $18.00	$ 252.00	
Independent claims	2 - 3 =	0	X $80.00	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 1242.00	
Reduction of 1/2 for filing by small entity, if applicable. Small Entity Statement verified by Applicant(s) attorney.				$ 0.00	
SUBTOTAL =				$ 1242.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ 0.00	
TOTAL NATIONAL FEE =				$ 1242.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 0.00	
TOTAL FEES ENCLOSED =				$ 1242.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ **1242.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Assistant Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0650. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Barbara E. Johnson
700 Koppers Building
436 Seventh Avenue
Pittsburgh, Pennsylvania 15219-1818
Telephone: (412) 471-8815
Facsimile: (412) 471-4094

Barbara E. Johnson
SIGNATURE
Barbara E. Johnson
NAME
31,198
REGISTRATION NUMBER

526 Rec'd PCT/PTO 02 JAN 2001

Form PTO-1390 (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 702-002201
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/720933

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATES CLAIMED
PCT/NL99/00417	02.07.99 (July 2, 1999)	02.07.98 (July 2, 1998)

TITLE OF INVENTION **BONE CEMENT WITH ANTIMICROBIAL PEPTIDES**

APPLICANT(S) FOR DO/EO/US **Elisabeth H. BURGER, Arie VAN NIEUW AMERONGEN, Paulus I. J. M. WUISMAN**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ *This is a* ***FIRST*** *submission of items concerning a filing under 35 U.S.C. 371.*

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

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4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ *is transmitted herewith (required only if not transmitted by the International Bureau).*
   b. ☒ has been transmitted by the International Bureau.
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   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ *A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).*

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

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13. ☒ A FIRST preliminary amendment.
    ☐ *A SECOND or SUBSEQUENT preliminary amendment.*

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:
    a. WO 00/01427-Front Page with Abstract, specification and claims (15 pp.)
    b. Search Report (4 pp.)
    c. International Preliminary Examination Report (4 pp.)

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/720933	INTERNATIONAL APPLICATION NO. PCT/NL99/00417	ATTORNEY'S DOCKET NUMBER 702-002201

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

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ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	34 - 20	14	X **$18.00**	$ 252.00	
Independent claims	2 - 3 =	0	X **$80.00**	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$ 0.00	
TOTAL OF ABOVE CALCULATIONS =				$ 1242.00	
Reduction of 1/2 for filing by small entity, if applicable. Small Entity Statement verified by Applicant(s) attorney.				$ 0.00	
SUBTOTAL =				$ 1242.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ 0.00	
TOTAL NATIONAL FEE =				$ 1242.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 0.00	
TOTAL FEES ENCLOSED =				$ 1242.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ **1242.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. ____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Assistant Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0650. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Barbara E. Johnson
700 Koppers Building
436 Seventh Avenue
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Telephone: (412) 471-8815
Facsimile: (412) 471-4094

Barbara E. Johnson
SIGNATURE
Barbara E. Johnson
NAME
31,198
REGISTRATION NUMBER

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 18 February 2000 (18.02.00)	
International application No. PCT/NL99/00417	**Applicant's or agent's file reference** L/VQ54/cst/1
International filing date (day/month/year) 02 July 1999 (02.07.99)	**Priority date** (day/month/year) 02 July 1998 (02.07.98)
Applicant BURGER, Elisabeth, Henriëtte et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

11 January 2000 (11.01.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Claudio Borton

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
LVQ54/CST/1		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 99/ 00417	02/07/1999	02/07/1998

Applicant

STICHTING SKELETAL TISSUE ENGINEERING

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. --

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00417

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L24/10 A61L27/22 A61K38/10 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39202 A (OSTEOGENICS INC) 12 December 1996 (1996-12-12) page 44, line 4; claims	1,32-34
A	EP 0 510 912 A (MORINAGA MILK INDUSTRY CO LTD) 28 October 1992 (1992-10-28) claims; examples	1-34
A	WO 97 18827 A (INTRABIOTICS PHARMACEUTICALS I) 29 May 1997 (1997-05-29) claims; examples	1
A	WO 94 15653 A (GENENTECH INC) 21 July 1994 (1994-07-21) claims	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1999	16/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 99/00417

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 20064 A (AMERICAN DENTAL ASS) 15 September 1994 (1994-09-15) claims; examples 1-10	1,32-34
A	DUCAN YU ET AL: "SELF-SETTING HYDROXYAPATITE CEMENT: A NOVEL SKELETAL DRUG-DELIVERY SYSTEM FOR ANTOBIOTICS" JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 81, no. 6, 1 June 1992 (1992-06-01), pages 529-531, XP000271282 ISSN: 0022-3549	1,32-34
A	WO 92 01462 A (SCRIPPS RESEARCH INST) 6 February 1992 (1992-02-06) claims	1
E	WO 99 37678 A (HELMERHORST EVA JOSEPHINE ;NIEUW AMERONGEN ARIE VAN (NL); STICHTIN) 29 July 1999 (1999-07-29) the whole document	1-34

1

INTERNATIONAL SEARCH REPORT

ormation on patent family members

International Application No
PCT/NL 99/00417

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639202 A	12-12-1996	AU 6149696 A	24-12-1996
		BR 9608344 A	05-01-1999
		CA 2223596 A	12-12-1996
		EP 0830149 A	25-03-1998
		JP 11506659 T	15-06-1999
EP 0510912 A	28-10-1992	AU 664697 B	30-11-1995
		AU 1514692 A	29-10-1992
		CA 2066997 A	25-10-1992
		DE 69223844 D	12-02-1998
		DE 69223844 T	16-04-1998
		DK 510912 T	09-02-1998
		JP 5148295 A	15-06-1993
		NZ 242437 A	27-07-1993
		US 5424396 A	13-06-1995
WO 9718827 A	29-05-1997	AU 704851 B	06-05-1999
		AU 1162997 A	11-06-1997
		AU 7739496 A	11-06-1997
		CA 2238429 A	29-05-1997
		CZ 9801591 A	14-10-1998
		CZ 9801592 A	16-12-1998
		EP 0862448 A	09-09-1998
		EP 0865292 A	23-09-1998
		HU 9901183 A	28-07-1999
		NO 982310 A	22-07-1998
		NO 982311 A	22-07-1998
		PL 326924 A	09-11-1998
		WO 9718826 A	29-05-1997
WO 9415653 A	21-07-1994	AT 153535 T	15-06-1997
		AU 671721 B	05-09-1996
		AU 6026294 A	15-08-1994
		CA 2151486 A	21-07-1994
		DE 69403439 D	03-07-1997
		DE 69403439 T	23-10-1997
		DK 679097 T	22-12-1997
		EP 0679097 A	02-11-1995
		ES 2105641 T	16-10-1997
		GR 3024277 T	31-10-1997
		JP 8505548 T	18-06-1996
		US 5422340 A	06-06-1995
WO 9420064 A	15-09-1994	US 5522893 A	04-06-1996
		AT 183382 T	15-09-1999
		AU 684722 B	08-01-1998
		AU 4923993 A	26-09-1994
		BR 9307825 A	14-11-1995
		CA 2157890 A,C	15-09-1994
		DE 69326082 D	23-09-1999
		EP 0688202 A	27-12-1995
		JP 8510713 T	12-11-1996
		US 5542973 A	06-08-1996
		US 5545254 A	13-08-1996
		US 5695729 A	09-12-1997
WO 9201462 A	06-02-1992	CA 2047317 A	20-01-1992
		JP 6504260 T	19-05-1994

INTERNATIONAL SEARCH REPORT

ormation on patent family members

International Application No
PCT/NL 99/00417

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201462 A		US 5294605 A	15-03-1994
WO 9937678 A	29-07-1999	NONE	